

## Anamnestic Protective Immunity to *Bacillus anthracis* Is Antibody Mediated but Independent of Complement and Fc Receptors<sup>▽</sup>

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**The threat of bioterrorist use of *Bacillus anthracis* has focused urgent attention on the efficacy and mechanisms of protective immunity induced by available vaccines. However, the mechanisms of infection-induced immunity have been less well studied and defined. We used a combination of complement depletion along with immunodeficient mice and adoptive transfer approaches to determine the mechanisms of infection-induced protective immunity to *B. anthracis*. B- or T-cell-deficient mice lacked the complete anamnestic protection observed in immunocompetent mice. In addition, T-cell-deficient mice generated poor antibody titers but were protected by the adoptive transfer of serum from *B. anthracis*-challenged mice. Adoptively transferred sera were protective in mice lacking complement, Fc receptors, or both, suggesting that they operate independent of these effectors. Together, these results indicate that antibody-mediated neutralization provides significant protection in *B. anthracis* infection-induced immunity.**

*Bacillus anthracis*, the etiological agent of anthrax, is a gram-positive, aerobic, spore-forming bacterium (25). Dormant spores are highly resistant to adverse environmental conditions and are able to reestablish vegetative growth in the presence of favorable environmental conditions (29). Anthrax is initiated by the entry of spores into the host through the skin, the gastrointestinal tract, or the respiratory epithelium after inhalation of airborne spores (10, 23, 27, 46). The inhalational form of anthrax is the most severe and is associated with rapid progression of disease and death (3, 11, 54). The best described virulence determinants of *B. anthracis* are encoded on two large plasmids (pXO1 [185 kb] and pXO2 [97 kb]) (20). The three genes that encode the proteins that combine to form the *B. anthracis* toxins (*cya*, *lef*, and *pag*) are found on the pXO1 plasmid (16–18). The combination of the *pag*-encoded protective antigen (PA) and the *cya*-encoded edema factor (edema toxin) causes edema when injected subcutaneously, and the combination of PA and the *lef*-encoded lethal factor (lethal toxin) causes death when injected intravenously (36). Capsule, composed of poly-D-glutamic acid, is encoded on the pXO2 plasmid. The capsule is believed to protect vegetative cells from microbicidal activity and serum proteins (14, 33, 48). Although the recent interest in *B. anthracis* pathogenesis is rooted in its potential as a bioterrorist weapon, it should be remembered that *B. anthracis* remains endemic throughout the world, and many people die yearly from anthrax due to environmental exposure. In many parts of the world anthrax outbreaks occur regularly in herds of wild and domestic animals (5, 37, 44, 45, 47). These outbreaks have environmental, as well

as economic impact, on the affected regions and provide a source of infection for the human population. In contrast to many pathogens that appear to be host limited, *B. anthracis* is able to efficiently infect and overwhelm the immune response of a remarkably wide range of hosts. Some aspects of its complex interactions with the host immune response have been partially illuminated by recent efforts to develop more effective vaccines.

Efforts to develop improved vaccines have focused on specific bacterial components. Since PA was shown to be the principle immunogen of the licensed vaccine (41, 51), it has been studied extensively as the primary component of numerous recombinant vaccine formulations. Antibodies to PA protect animals against lethal disease, although other antigens may also contribute to protective immunity (4, 8, 21, 24, 30, 32, 50, 53). Fab fragments recognizing PA have been shown to be protective, suggesting that antibody neutralization of PA is sufficient to protect against lethal disease (26, 32, 34, 52). In addition to understanding the host response to vaccination, there is significant value in increasing our understanding of the biology of the anthrax organism, including its complex interactions with the host immune response. In particular, identifying mechanisms involved in protective immunity following infection, which may be different from those induced by current vaccination approaches, could have important applications.

Antibodies can function by three main mechanisms: complement activation, opsonization for FcR-mediated phagocytosis, or neutralization, which refers to antibodies' ability to interfere with pathogen functions simply by binding. Antibody-mediated clearance of bacterial pathogens can require any one, or combinations, of these activities. For example, bacteria in the lungs can be unaffected by antibodies in the absence of complement components or FcRs, indicating that a complex combination of Fc-associated effector functions is required for

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bacterial clearance (22). Although neutralization is likely to be the mechanism by which PA-based vaccines work, it is not clear that *B. anthracis* infection-induced immunity provides subsequent protection by the generation of anti-PA antibodies. Also, it is not clear whether anti-PA antibodies contribute to a reduction in bacterial numbers during an infection. Therefore, the mechanisms of protection elicited by PA vaccine-induced immunity, which protects against toxin-mediated pathology, are likely to differ from those that are induced by infection with viable spores.

*B. anthracis* toxins can interfere with innate, inflammatory, and adaptive immune responses at various levels. Lethal toxins can kill or inactivate immune cells such as monocytes, macrophages, and neutrophils (2, 7, 39, 42). Edema toxin can hinder lipopolysaccharide-induced cytokine production by macrophages (19). By suppressing activation of macrophages or dendritic cells, *B. anthracis* toxins may interfere with antigen presentation pathways involved in the generation of adaptive immunity (1). Furthermore, anthrax toxins have been shown to act directly on adaptive immune cells, blocking multiple kinase signaling pathways involved in T-cell activation (6, 38). Treating mice with toxins alone has been shown to inhibit the ability of T cells to proliferate and secrete cytokines. Thus, *B. anthracis* can manipulate host immunity at various levels, some of which appear to be dependent on complexities of local concentrations of bacteria, toxins, and various immune cells. These complex interactions between host and bacterial components cannot be simulated in vitro or with purified bacterial components and/or toxins in vivo but are best studied in the context of infection.

Here we explore the immunological mechanisms involved in the generation of *B. anthracis* induced immunity after aerosol exposure to spores. We have taken the approach of experimentally infecting immunodeficient mice to determine which immune factors are required for the generation of protective anamnestic immunity. Our results indicate that both B and T cells were required, which is probably attributable to their respective roles in the induction of antibody production. T-cell-deficient mice failed to produce significant levels of immunoglobulin G (IgG) antibody to PA, and the adoptive transfer of anti-*B. anthracis* serum was sufficient for protection against challenge. Adoptively transferred antibodies were protective in mice lacking both complement and FcRs. Together, these data indicate that protective immunity induced by toxigenic, non-encapsulated *B. anthracis* infection acts via an antibody-dependent mechanism that does not require antibody Fc effector functions.

#### MATERIALS AND METHODS

**Generation and purification of *B. anthracis* spores.** Spores were prepared from *B. anthracis* strain 7702 (pXO1<sup>+</sup>, pXO2<sup>-</sup>) by using the method described by Finlay et al. (9). Purification of spores was performed using 58% (vol/vol) renografin (Renocal-76 diluted in distilled water [dH<sub>2</sub>O]; Bracco Diagnostics, Princeton, NJ) prior to use. Spores were layered on the 58% renografin and were spun at 4,000 × g for 30 min in a swinging-bucket rotor. The spore pellet was washed twice with dH<sub>2</sub>O (6,000 × g for 30 min), and the spores were resuspended in dH<sub>2</sub>O. The final concentration of the stock spore solutions was adjusted to 5 × 10<sup>9</sup> spores/ml.

**Spore challenge of mice.** A/J, C57BL/6, RAG2<sup>-/-</sup>, μMT, and TCR<sup>-/-</sup> mice were obtained from Jackson Laboratory (Bar Harbor, ME). FcγRI/FcγRIII/FcεRI-deficient mice were obtained from Taconic Laboratories (Germantown, NY). Aerosol challenges were performed as previously described (31, 40).

Briefly, mice were exposed to aerosolized spores prepared from *B. anthracis* strain 7702 for 90 min. At 1 h after exposure, four mice were euthanized, and their lungs were homogenized and plated to confirm that doses between 2 × 10<sup>6</sup> and 4 × 10<sup>6</sup> per mouse were delivered (average retained dose). Intraperitoneal (i.p.) challenges were performed as follows. Mice were injected i.p. with 10<sup>9</sup> spores prepared from *B. anthracis* strain 7702 suspended in 100 μl of sterile dH<sub>2</sub>O. For complement depletion studies, 24 h prior to challenge, mice were injected i.p. with two 5-U doses of cobra venom factor (CVF) given 4 h apart. For passive protection studies, mice were injected i.p. with 100 μl of convalescent mouse anti-Ba serum or with mouse normal serum prior to challenge.

**Production of anti-Ba polyclonal serum.** C57BL/6 mice were challenged with *B. anthracis* Sterne strain spores by aerosol as described above, and survivors were rechallenged on day 14 postchallenge. Sera were collected from mice 2 weeks after the second challenge. The pooled convalescent mouse serum had an enzyme-linked immunosorbent assay (ELISA) dilution endpoint titer of 10<sup>5</sup>. Convalescent-phase serum and normal serum were heat treated at 56°C for 30 min to inactivate complement.

**Measurement of serum antibody titers.** Total serum IgG antibody titers to PA were determined by using a quantitative anti-rPA ELISA. Ninety-six-well microtiter plates (Immunolon 2HB; ThermoLabsystems, Franklin, MA) were coated with 100 μl of recombinant PA (1 μg/ml)/well overnight at 4°C. Plates were then washed (phosphate-buffered saline plus 0.05% Tween) and blocked with 3% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C. Plates were incubated with 100 μl of serially diluted (1:100 to 1:300,000; in blocking buffer) serum samples at 37°C for 1 h. Plates were then incubated for 30 min at room temperature with purified horseradish peroxidase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD) diluted 1:1,000 in blocking buffer. Finally, the plates were incubated for 15 to 20 min at room temperature with 100 μl of ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] substrate (KPL). The reaction was stopped by adding 100 μl of ABTS peroxidase stop solution (KPL). Absorbance values were obtained by using a Molecular Devices (Sunnyvale, CA) VERSA<sub>max</sub> microplate reader at 405 nm. Samples were assayed in triplicate, and the endpoint antibody titers were expressed as the maximum dilution giving an absorbance of >0.2 (405 nm). The results are presented as the reciprocal of the dilution multiplied by the absorbance value. Serum antibody titers to whole-cell antigens were similarly determined, except that the ELISA plates were coated with 100 μl of 2.5 × 10<sup>7</sup> cells/ml heat-killed (45 min at 65°C) *B. anthracis* strain 7702.

#### RESULTS AND DISCUSSION

Respiratory infections often induce an immune response that is different from that induced by vaccines delivered parenterally. Furthermore, the use of a small number of factors in vaccines would be predicted to induce antibodies that may neutralize these factors, and thereby reduce pathogenesis, but may not have direct antimicrobial activity. The immune response generated during infection is generally quite effective in eliminating the foreign agent and includes many different efficient antimicrobial activities. For example, T cells can lyse infected cells as well as recruit and activate phagocytic cells. Antibodies generated during bacterial infection can bind to the bacterial surface and have potent effector functions that can lead to bacterial lysis via complement, or facilitate phagocytosis by immune cells via Fc receptors (FcRs). Bacterial respiratory pathogens have been shown to induce immunity that is dependent on T cells or on specific antibody effector functions and can even be dependent on the combination of both antibody effector functions and T cells (35). Considering that *B. anthracis* has mechanisms to affect T-cell functions that could disrupt the generation of various immune functions, it is important to understand the mechanism of anamnestic immunity to this pathogen.

Antibodies are necessary for protective immunity to *B. anthracis*. We set out to establish a model in which animals could be immunized via respiratory infection and subsequently assessed for protective immunity against *B. anthracis* challenge.

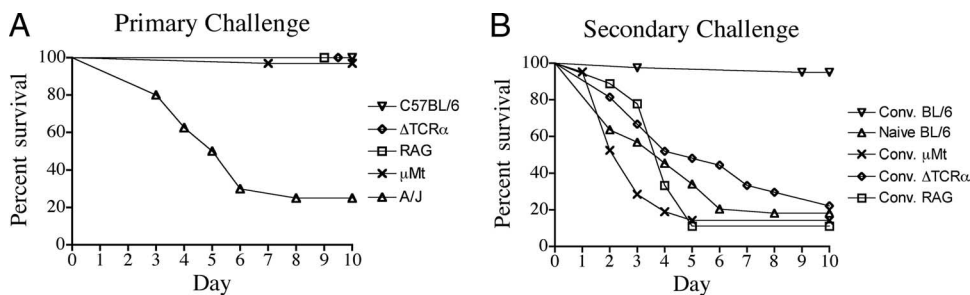


FIG. 1. Primary (A) and secondary (B) challenge of immunodeficient mice. Naive mice were exposed to aerosols of spores prepared from *B. anthracis* strain 7702 as described in the text. Survival of groups of mice with retained doses between  $2 \times 10^6$  and  $4 \times 10^6$  spores after aerosol challenge is shown. For each strain, the cumulative mortality of three independent challenges is represented in the graph ( $n = 30$ ). Convalescent mice that survived the primary challenges were subsequently challenged with  $10^9$  spores of strain 7702 injected by the i.p. route 4 weeks after the original exposure. In each experiment, a group of naive C57BL/6 mice was included as a positive control for the challenge. The survival of each group is shown. For each mouse strain, the cumulative mortality of three independent challenges is represented in the graph ( $n = 30$ ), except for the  $\mu MT$  group, for which one mouse died in the primary challenge ( $n = 29$ ).

This model required conditions under which mice would survive an initial infection to generate protective immunity. It also required the use of sensitive mice in which generated immunity, or adoptively transferred immunity, could measurably affect survival. We used C57BL/6 mice, which normally survive aerosol challenge with  $2 \times 10^6$  to  $4 \times 10^6$  spores of the Sterne strain of *B. anthracis* but are susceptible to i.p. challenge with  $10^9$  spores. This allowed us to assess the protection conferred by adaptive immunity induced by aerosol infection. Since the Sterne strain is acapsular, it is susceptible to the effects of the complement cascade. We have previously shown that the ability of C57BL/6 mice to survive aerosol challenge with the Sterne strain is dependent on complement and is abrogated by complement depletion by genetic or pharmacological means (15). A/J mice are naturally complement defective and sensitive to Sterne challenge. Using this combination of mice we can induce protective immunity to respiratory challenge in resistant mice (C57BL/6) and then assess adaptive immune protection against infections that would be lethal in naive mice (A/J).

To determine which adaptive immune functions might be required to control an initial *B. anthracis* infection, we exposed resistant C57BL/6 mice and C57BL/6 mice lacking B cells ( $\mu MT$ ), TCR $\alpha/\beta$  T cells (TCR $\alpha^{-/-}$ ), or both B cells and T cells

(Rag2 $^{-/-}$ ) and susceptible A/J mice. After aerosol challenge of A/J mice, spores disseminate to the draining lymph nodes where the appearance of vegetative bacteria are first observed (13, 31). Subsequently, bacilli are found in high numbers throughout the animal including the lungs, liver, heart, and spleen (31). After aerosol challenge of C57BL/6 mice, spores disseminate to the draining lymph nodes, where vegetative bacteria are observed, but the infection does not disseminate beyond the lymph nodes, and animals ultimately clear the infection and survive (data not shown). In our experiments, after aerosol challenge A/J mice succumbed ( $>70\%$ ) to infection within a week of challenge with *B. anthracis* Sterne strain (Fig. 1A). There was no mortality among the complement-sufficient mice, including all knockout strains, indicating that adaptive immunity is not required to survive primary aerosol challenge with Sterne strain. We then tested the ability of the convalescent C57BL/6,  $\mu MT$ , TCR $\alpha^{-/-}$ , and Rag2 $^{-/-}$  to survive a lethal i.p. challenge with Sterne-strain spores 4 weeks after the primary aerosol exposure. Naive C57BL/6 mice succumbed ( $>70\%$ ) within a week, indicating that a lethal i.p. dose was delivered (Fig. 1B). Convalescent C57BL/6 mice did not succumb to infection, indicating that prior aerosol infection induced immunity that protected these mice from lethal *B.*

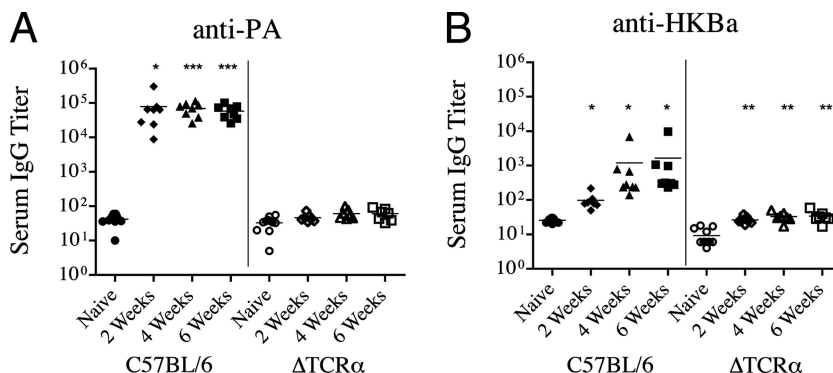


FIG. 2. Serum IgG levels in T-cell-deficient mice. C57BL/6 and TCR $\alpha^{-/-}$  mice were exposed to aerosols of spores prepared from *B. anthracis* strain 7702 as described in the text. Prior to exposure and 2, 4, and 6 weeks after exposure, mice were bled and serum titers against PA (anti-PA) (A) and heat-killed *B. anthracis* cells (anti-HKBa) (B) were determined as described in Materials and Methods. Statistical significance was determined by using Student *t* test analysis. In all cases, means were compared to those of the naive control group (\*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ ).

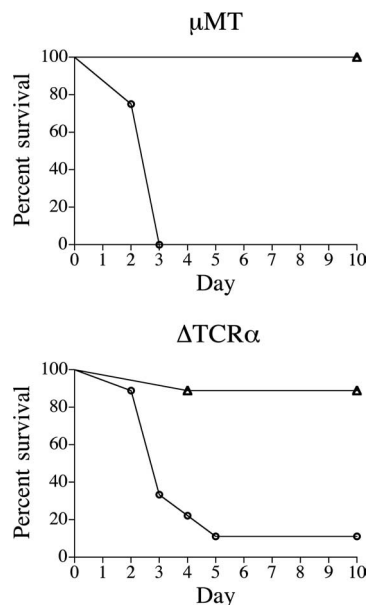


FIG. 3. Passive protection of convalescent-immunodeficient mice with anti-*B. anthracis* sera.  $\mu$ MT and  $\text{TCR}\alpha^{-/-}$  mice were exposed to aerosols of spores prepared from *B. anthracis* strain 7702 as described in Materials and Methods. At 4 weeks after primary challenge, all mice were challenged with  $10^9$  spores of strain 7702 delivered i.p. Immediately prior to rechallenge, half of each group of convalescent mice received mouse anti-Ba serum ( $\Delta$ ) and the other half received normal mouse serum ( $\circ$ ). The survival of each group is shown ( $n = 9$ ).

*anthracis* challenge. In contrast, convalescent  $\text{Rag}^{-/-}$  and  $\mu$ MT mice succumbed to infection ( $>70\%$ ), indicating that B cells are required for protective immunity and that T cells alone (which are present in  $\mu$ MT mice) are not sufficient for anamnestic protection. Interestingly,  $\text{TCR}\alpha^{-/-}$  mice also succumbed to secondary infection, indicating that T cells are required for initiating protective anamnestic responses.

Since T cells can affect immunity in several ways, including the efficient generation of various antibody isotypes, we examined whether  $\text{TCR}\alpha^{-/-}$  mice are defective in antibody responses to *B. anthracis* respiratory challenge. Sera, recovered from mice 2, 4, and 6 weeks after aerosol challenge, were analyzed by ELISA for IgG antibodies against whole heat-killed *B. anthracis* cells (anti-HKBa) or against protective antigen (anti-PA) (Fig. 2). C57BL/6 mice had elevated titers

recognizing heat-killed *B. anthracis* by week 2 that were increased at week 4, and very high titers of antibodies to PA by 2 weeks. In contrast, anti-HKBa and anti-PA titers in  $\text{TCR}\alpha^{-/-}$  mice were not significantly different from mock-challenged mice even 6 weeks after aerosol challenge. These data indicate that T-cell-deficient mice are defective in generating antibodies against *B. anthracis*, possibly explaining their defect in protective anamnestic immunity to secondary infection.

Taken together, the above data indicates that antibodies are required for protective anamnestic immunity to *B. anthracis*. To determine whether antibodies alone are sufficient to protect B-cell- and T-cell-deficient mice from lethal infection, we collected convalescent-phase serum from C57BL/6 mice that survived aerosol challenge with *B. anthracis* spores and transferred this polyclonal mouse anti-*B. anthracis* serum (anti-Ba serum) into  $\mu$ MT and  $\text{TCR}\alpha^{-/-}$  mice. These mice were subsequently challenged by the i.p. route with *B. anthracis*. This challenge killed 100% of naive C57BL/6 mice given normal serum (data not shown). The anti-Ba serum protected  $\mu$ MT and  $\text{TCR}\alpha^{-/-}$  mice, indicating that neither B cells nor T cells are required when anti-Ba antibodies are passively provided (Fig. 3).

**Antibody-mediated protection requires neither complement nor FcRs.** Antibodies can affect bacterial infections by various mechanisms, with effector functions including FcR-binding and/or complement activation. To determine whether complement activation was involved in the protection conferred by adoptively transferred immune sera, we performed an experiment similar to that described above by treating complement-deficient mice with convalescent-phase sera. This was done with A/J mice, which are naturally C5 deficient, and C57BL/6 mice treated with CVF to deplete complement. CVF enzymatically degrades complement and has been shown to result in near-complete depletion of complement activity (28, 43, 49). We have shown previously that the acapsular Sterne strain is virulent in complement-deficient and CVF-treated mice, so the aerosol challenge (instead of the i.p. challenge) can be used to assess protective immunity (15). A/J and CVF-treated C57BL/6 mice were treated with either mouse naive serum or anti-Ba serum and challenged with  $2 \times 10^6$  to  $4 \times 10^6$  spores by the aerosol route. As expected, the majority of A/J mice and CVF-treated C57BL/6 mice given naive serum succumbed to infection (Fig. 4). In contrast, most A/J mice and CVF-treated C57BL/6 mice given anti-Ba convalescent-phase serum survived challenge (Fig. 4). These data indicate that antibodies

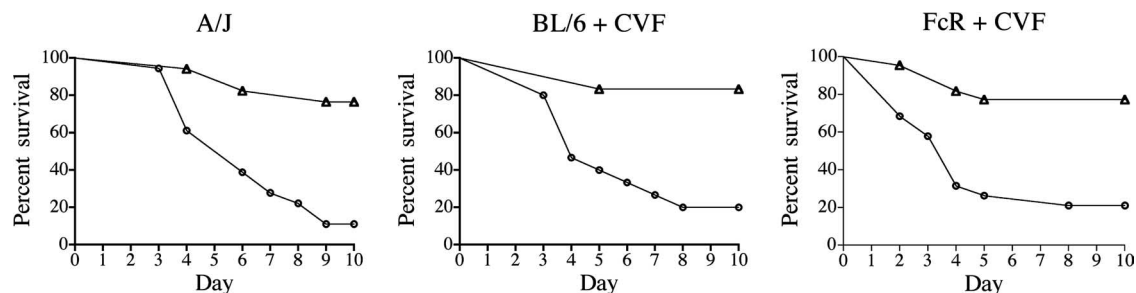


FIG. 4. Passive protection of complement-deficient and FcR-deficient mice. A/J mice, CVF-treated C57BL/6 mice, and CVF-treated  $\text{FcR}^{-/-}$  mice were given mouse anti-Ba serum ( $\Delta$ ) or normal mouse serum ( $\circ$ ) and challenged with  $2 \times 10^6$  to  $4 \times 10^6$  spores of strain 7702 delivered by aerosol. The survival of each group is shown. For each strain, the cumulative mortality of three independent challenges is represented in the graph ( $n = 18$  for each A/J group,  $n = 13$  for each BL/6 group, and  $n = 22$  for each  $\text{FcR}^{-/-}$  group).



are effective in protecting mice from lethal aerosol challenge with *B. anthracis* Sterne spores even in the absence of complement. Thus, anti-*B. anthracis* antibodies are able to act via a complement-independent mechanism.

To examine the role of FcRs in antibody-mediated anamnestic immunity to *B. anthracis*, naive or immune serum was transferred into CVF-treated FcR<sup>-/-</sup> mice. Since these mice were depleted of complement, they were susceptible to aerosolized *B. anthracis* Sterne. However, mouse anti-Ba serum was effective in protecting these mice from lethal aerosol challenge, indicating that serum antibodies require neither complement nor FcRs to effectively protect mice against lethal *B. anthracis* respiratory challenge (Fig. 4).

The results from our study show that a protective adaptive immune response is elicited in animals that survive an aerosol exposure to *B. anthracis* spores and that antibody production is required for this infection-induced protective immunity to *B. anthracis*. The data further demonstrate that anti-Ba serum alone, when adoptively transferred, are sufficient for protection to *B. anthracis* challenge. Transferred antibodies were protective even in the absence of complement, FcRs, or both, indicating that protection is not dependent these antibody effector functions. Thus, protection is based on antibody neutralization, likely by neutralizing factors such as the lethal, edema toxins or other components. These findings are consistent with what is known about the mechanisms of immunity induced by vaccines containing PA. Previous studies have shown that Fab fragments of antibodies induced by vaccination are sufficient for protection (26, 32, 34, 52), indicating that complement and FcR-binding activities of the antibody Fc domain are not required.

While these results indicate that neither complement nor FcRs are necessary for antibody-mediated protection, they do not rule out the possibility that either, or both, contribute. It is difficult to rigorously test other possible contributions of antibody effector functions, since it is not possible to do the converse of these studies, such as eliminating antibody neutralization function without affecting other antibody functions. In the work presented here we examined the mechanism of antibody-mediated protection induced after aerosol infection with a nonencapsulated, toxigenic strain of *B. anthracis*. It is possible that cellular immunity contributes to protection after aerosol challenge but alone is not required for survival to primary aerosol challenge. Previous work has indicated a role for cellular immunity in survival after vaccination with inactivated spores, and the authors of an earlier study noted protection to encapsulated, nontoxigenic *B. anthracis* challenge (12). However, these authors did not examine the survival of toxigenic, nonencapsulated strains after vaccination with inactivated spores. Thus, it is unclear whether the immunity induced by inactivated spores is relevant to encapsulation or is more indicative of a response to spore-specific antigens. It is possible that cellular responses contribute to immunity induced by vaccination with inactivated spores, but further work is required to outline the contribution of cellular immune responses in survival after aerosol challenge. Again, immunity induced to vaccination may be different than that induced after infection. Together, our results suggest that, after aerosol infection with a toxigenic strain of *B. anthracis*, the primary mechanism of

protective immunity involves the generation of neutralizing antibodies.

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